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13. ABSTRACT (Maximum 200 Words)

PARP is a chromatin bound enzyme, which is pivotal in the recovery of cells from DNA strand break damage. **SM** confers damage to cells causing DNA strand breaks. In collaboration with intramural scientists of USAMRICD, we recently demonstrated that **SM**, in part, causes cellular pathology and death via initiating apoptosis, and in primary PARP (+/+) fibroblasts by causing such an extensive depletion of ATP and NAD that the skin cells die by necrosis. Given that the mitochondria is a potential target of **SM** toxicity via release of cytochrome-c leading to activation of the downstream caspase cascade, most emphasis during year one has focused on the changes that occur in mitochondria following exposure to **SM**. We are also determining the role of PARP in **SM**-induced necrosis and apoptosis and why this response is cell-type specific. **SM** induces apoptosis in both wild type and PARP(-/-) keratinocytes, and apoptosis in PARP(-/-) fibroblasts, thus, we will determine the targets in the apoptotic cascade "upstream". Proteins and biological systems to be surveyed include: the Bcl-2 family; p53; depletion of NAD/ATP levels; table transfection of a mutant PARP cDNA (catalytically inactive chromatin and nuclear structure/ ultrastructure during early apoptosis. We anticipate also using our PARP K/O mice. Given that PARP(-/-) fibroblasts undergo **SM**-induce apoptosis, we will test whether there is also an initial decrease in the NAD/ATP levels in PARP(-/-) fibroblasts after exposure to SM, possibly due to other genes in the "PARP family".

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(Research From May 1, 2002- April 30,2003)

Introduction:

Overall, significant progress was made during Year One. Due to staffing issues during the first year of the contract a number of the objectives initially presented in the SOW had to be delayed. Additionally, because of the extreme delayed starting date (due to 9/11) and reduced funding support in recent years these objectives may not be practical, nor still relevant. However, utilizing the resources available to me, I have made excellent progress on setting up a new cell system (Jurkat cells) and identifying a profitable research direction looking at the role of oxidant related cell injury by mustards. For the second year of the Contract, after a recent site visit with Dr.. William Smith, my COR, I plan to extend the research currently being pursued to determine if the protection caused by SM of ROS damage in Jurkat cells will be also seen in 1) a respiratory (rat airway epithelium) model, and 2) a skin (keratinocyte cell line, probably NCO or HaCat) model.

Additionally, during the second year I will be directing my research to points of study that apply to PARP and continue, as in the past, to elucidate if PARP and poly ADP-ribosylatiohas an active role in the mechanisms of SM pathology, I was lead to appreciate that the USAMRAA had been quite interested in studies which **had a high likelihood of providing <u>direct protection</u> against the pathology to military personal exposed to SM.** At this same time I had begun experiments, which seemed initially quite promising, and indicated that SM was, in part causing a significant increase in cellular Reactive Oxygen species (ROS). Preliminary experiments indicated that natural antioxigents seemed to protect cells against SM –induced death. After discussions with my COR, we began to direct most of our SOW to this topic for year one of the contract. It should be noted that several of the original objectives of the amended Contract were, in fact, directly related to ATP/NAD levels in cells, and stresses placed on mitochondria membrane potentials; nicely complements this new work initiated in year one.

Since at that time we also began to experience difficulties with the growth of our various skin cells, which had been used successfully in the past.

SM induced ROS, protection by Antioxidants

Accordingly, we began a detailed study on Protective roles of ant-oxidants in Jurkat T-cells on sulfur half mustard (CEES). A manuscript on the first year's work on this topic was recently submitted to *The British Journal of Pharmacology*, and the data from this paper constitute the bulk of the Report for this first year. Essentially in this work we investigated the biochemical changes characteristic of apoptosis and subsequent mechanism of CEES toxicity in Jurkat T cells in order to associate and identify rational, therapeutic, protective mechanisms against this toxicity. A significant reduction in intracellular concentrations of GSH was observed in Jurkat T cells almost immediately after exposure to CEES. This effect was accompanied by increased intracellular ROS generation and by loss of mitochondrial membrane potential that declined slowly at 5 h and more markedly by 12 h. Additionally, L-BSO, an inhibitor of an early step of GSH synthesis, increased CEES induced cell death.

Additionally, CEES exposure in Jurkat cells induced biochemical markers of apoptotic cell death such as: PARP cleavage, caspase-3 processing and activity. The pro-oxidant effect of CEES was inhibited by either exogenously added thiol-containing antioxidant NAC or GSH-ethyl ester. Accordingly, CEES toxicity appears to be mediated, in part, by generation of ROS and depletion of GSH. Since sulfur mustard is still a potentially active biohazard, the protective effects of the various antioxidants against certain types of toxicity of a derivative of this agent in Jurkat cells may be useful to provide approaches to counteract mustard toxicity.

I have experienced unforeseen difficulties concerning personnel in my laboratory, which I discussed with my COR. This has resulted in a lesser amount of progress than anticipated. I had originally assigned the direction and execution of the majority of the experiments on this contract to a Post Doctoral Research Fellow- Dr. Suhua Han. Only after a few months into Year One, she unexpectedly became pregnant. Additionally, in her first trimester there were some medical complications, which required her to have bed rest for an extra month. Since she has faculty status, a significant amount of her salary while she has been on maternity leave was continued to be paid from the contract budget. I could not afford to hire a temporary replacement. Dr. Han just recently returned, with only a few months of Year One remaining. Additionally, a laboratory technician, who had also been working on the Army SM project, is also now taking maternity leave until mid June 1.

When all his occurred, I discussed it with my COR, Dr. William Smith and he suggested I report this to my Contracting Officer (which I did). As much as they could afford their time from responsibilities on other projects and grants. Based upon my long-term association with USAMRAA and having fully met my contractual terms since 1990, I want to assure USAMRMC, as in the past, I will complete those aspects of the research, which I feel useful at this time, with emphasis on the new exciting direction of anti-oxidant protection of SM damage. The latter results will be summarized below in this Annual Report.

BODY

STATEMENT OF WORK

SOW 1.3. Mitochondrial changes:

Description of Work Accomplished:

This work thus constitutes the majority of the research work that was accomplished by my laboratory during the first year of this two-year contract, and will be reported in detail below. It is important to note that the results and the execution of these types of experiments were reported, in total, in the first three quarterly reports as required in the contract.

[Reference citations and Experimental Methods are found in the Appendix.]

The sulfur mustard derivative 2-chloroethylethyl sulfide (CEES) is a valid biochemical analog of sulfur mustard since it causes, albeit, limited vesication but alkylates a wide range of biological molecules. SM is absorbed into the general circulation at sites of direct contact, such as the skin, eyes and airways, and those organs are the most commonly effected.

Hematological complications such as leucopoenia and bone marrow depletion are observed in humans or rats after being poisoned by sulfur mustards, indicating that leukocytes are also targets of sulfur mustards. Recent studies on CEES have shown that exposure of a Jurkat T cell line to CEES induces biochemical and morphological changes that are characteristic of apoptotic cell death (Zhang et al., 2002). This is one reason why we chose Jurkat T cells for out initial studies during the first year of this contract. The effects shown in

T cells included chromatin condensation, degradation of genomic DNA into both high molecular weight and oligonucleosomal fragments, up-regulation of caspases 3, 6, 8 and 9 (Many of these topics have been included in aims that were intentionally not pursued in the first year protocols of my SOW, since they had already been performed by Zhang and co-workers are thus available to the current work we have performed during the first year). The above workers showed that CEES exposure Jurkat cells causes down regulation of the transcription of Akt, protein kinase B, which normally inhibits apoptosis.

One major initial factor, which contributes to the commitment of cells to apoptotic death, is the alteration of mitochondrial function and the consequent loss of mitochondrial membrane potential ($\Delta\Psi_{mito}$) (Mancini *et al.*, 1997; Salvioli *et al.*, 1997). Our lab in the past has recently performed a number experiments measuring the effects of toxic agents other than mustards on mitochondrial membrane potentials and also effects of antioxidants and protection. The perturbation of mitochondrial function during earlier apoptosis increases generation of reactive oxygen species (ROS) (Jabs 1999), which results in an oxidative environment that contributes to the apoptotic death process, in part, by depleting cellular antioxidants. Based on these past studies with other toxic agents, we were fascinated by the possibility that thiol anti-oxidants would be highly effective in rescuing cells from toxic cell death. Given that the toxicity of SM and concomitant generation of ROS has been proposed to result from electrophilic or oxidative stress. During the first year our results have been quite encouraging in showing that oxidative stress of CEES toxicity in Jurkat T cells as well as the protective properties of GSH and NAC against CEES were quite pronounced and constitute possibly a beneficial effect for military personnel. ***[However, as recently pointed out to me (4/29/03) by Dr. Smith, this may not be true in skin or skin-derived cells., which utilizese glycolysis, rather mitochondrial oxidation for ATP generation.***

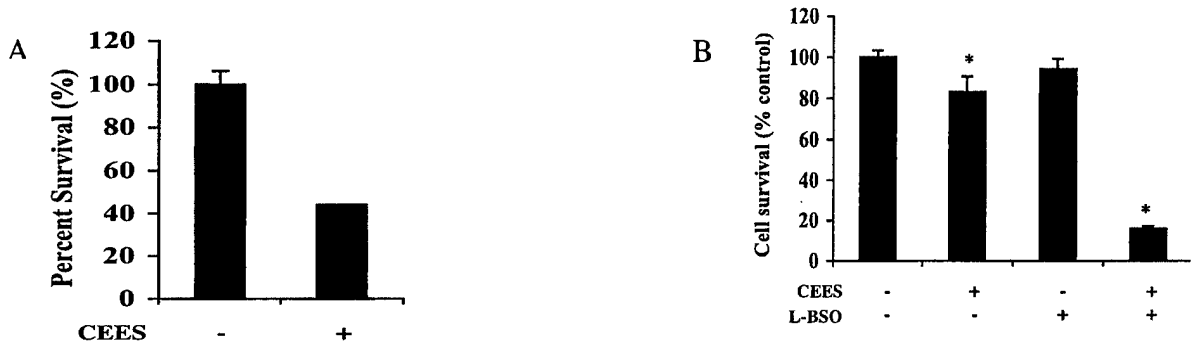
The detailed methods for these experiments provided in the **appendix**, included with the application (*Appended publication*).

CEES–induced death in Jurkat T cells is affected by GSH depletion

As noted, recent study has shown that exposure of Jurkat T cells to CEES induces apoptotic cell death (Zhang et al. 2002). In our experiments incubation of Jurkat T cells for

18h with CEES caused an approximate 60% loss of cell viability, as assessed by calcein-AM staining (Figure 1A).

Figure 1. The influence of the depletion GSH on CEES-induced death in Jurkat T cells



Jurkat T cells were incubated for 18 h in the absence (control) or presence of a 600 uM CEES, after which cell viability was assessed by measurement of calcein-AM fluorescence.

Jurkat T cells were incubated initially for 20 h in the presence or absence of 200 mM L-BSO. Subsequent incubation for 6 h with or without CEES in the continued presence or absence of L-BSO was

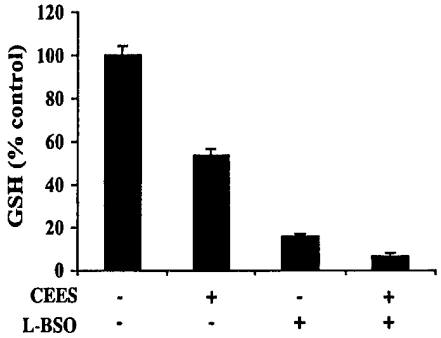
The intracellular concentration of GSH has shown to be altered by a number of toxic agents (Hathway 2000). Accordingly, an investigation of whether the concentration of GSH is of consequence to CEES toxicity in Jurkat T cells was performed next. In order to deplete cells of GSH, Jurkat T cells were incubated for 20 h with 200 μM L-BSO, an agent which is well established to selectively inhibit γ-glutamylcysteine synthetase, which represents the rate-limiting enzyme in GSH biosynthesis (Chiba *et al.*, 1996; Oda et al. 1999). Significantly, L-BSO reduced Jurkat T cell GSH levels to 83% of untreated cells.

We then treated the cells with CEES for 6 h and then cell viability was measured by calcein-AM staining. We found that the inhibition of GSH synthesis by L-BSO significantly potentiated CEES–induced cell death (Figure. 1B). While CEES alone resulted in an ~20% lowering of cell viability, the combination of CEES and the GSH inhibitor, L-BSO, resulted in >80% cell death. In the case of Jurkat cells, L-BSO alone induced a 20% decrease in cell viability in the absence of the sulfur mustard derivative (Figure 1B). We feel that the above experiments provided evidence that the intracellular concentration of GSH is of importance in the CEES toxicity observed in Jurkat T cells.

Accordingly, during YEAR ONE, we examined the direct effect of CEES exposure was examined with respect to the intracellular levels of GSH by measurement of this metabolite in Jurkat T cells after 6 h exposure to the sulfur mustard derivative.

Figure 1.

C



Intracellular concentrations of GSH in Jurkat T cells upon exposure to CEES and L-BSO. Jurkat T cells were incubated first for 20 h in the presence or absence of 200 mM L-BSO, and then for 3 h with or without CEES in the continued presence or absence of L-BSO. Cell extracts were then assayed for GSH.

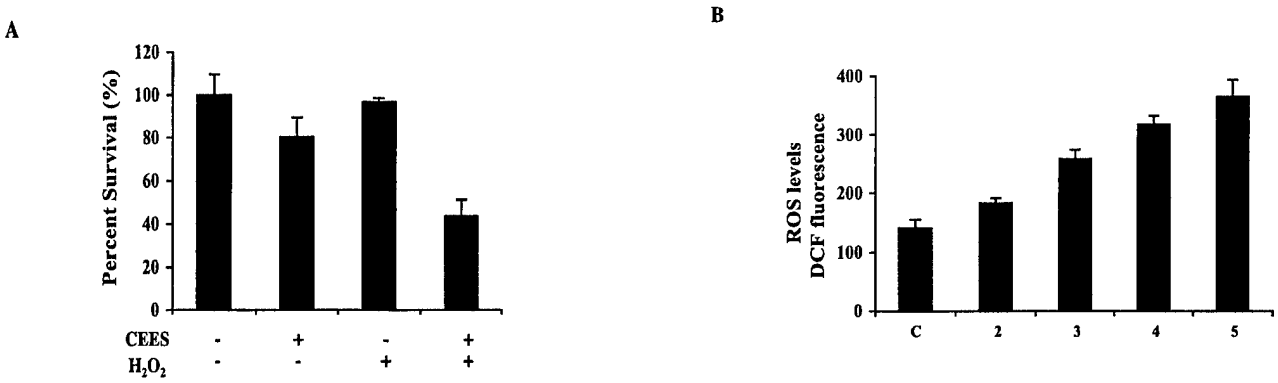
CEES markedly reduced the level of GSH (~50%) during this 6 h time exposure (Figure 1C). L-BSO potentiated the lowering of GSH by CEES to 10% of the control. We feel that this data, obtained during year 1 emphasizes the important role of GSH level perturbation by an inhibitor such as L-BSO in the overall cytoxicity caused by CEES.

CEES–induced cell death and influence of oxidative stress.

We reasoned that the generation of an oxidative intracellular environment presumably resulted from the increased levels of ROS as occasioned by the chemical reactivity of the sulfur mustard derivative was directly related to the decrease in intracellular GSH levels. Accordingly we tested whether several thiol anti-oxidants such as GSH and its precursor NAC resulted in a protective effect against CEES-induced cell toxicity and death. To test this hypothesis, we first examined the effect of a low concentration of H_2O_2 on CEES–induced death in Jurkat cells. The cells were pretreated with 25 μM H_2O_2 for 1 h, subsequently,

incubated with CEES for 6 h in the continued presence of H_2O_2, and then assayed for viability by calcein-AM staining. We found it significant that treatment of the cells with H_2O_2 alone had no significant effect on cell viability (Figure 2A), however, while CEES alone induced approximately 20% loss of cell viability, **the combination of CEES and H_2O_2 resulted in greater than 50% cell death**

Figure. 2 ***CEES-induced cell death: effect of oxidative stress***



Jurkat T cells were incubated first in the absence or presence of 25 μM H2O2 for 1 h and then with or without 600 uM CEES for 6 h (in the continued presence or absence of H2O2), after which cell viability was assessed by measurement of calcein-AM fluorescence.

CEES-treated cells at the indicated times demonstrate progressively increasing ROS generation as compared with the controls.

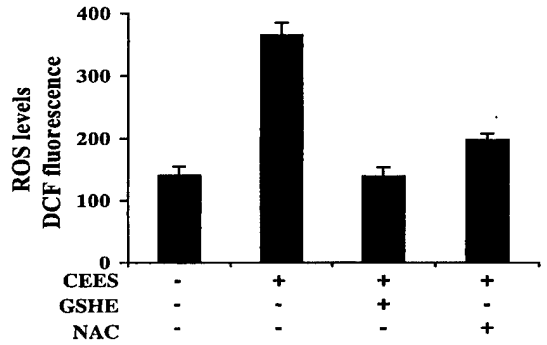
Direct Flourometric evidence for CEES generated heightened ROS in cells

The data shown above (Figure. 1C, 2B) indicated a synergistic effect of H_2O_2 on CEES killing further suggested that the sulfur mustard analog was presumably acting by a direct generation of active oxygen species in cells. The latter was directly measured by oxidation of H2DCF to the fluorescent DCF moiety, which is measured flourometrically and is a valid indicator of the presence of ROS synthesis in cells. The data in Figure 2B above showed that CEES alone dramatically increased the levels of ROS in Jurkat cells by 2 fold at a 5 h time course over the control cells. However preincubation of the cells with GSH and NAC significantly reduced the induced ROS generation. These results corroborate that the observed

increase in ROS formation is indeed due to a pro-oxidant cellular activity of CEES (see figure. 2C below).

Figure. 2

C

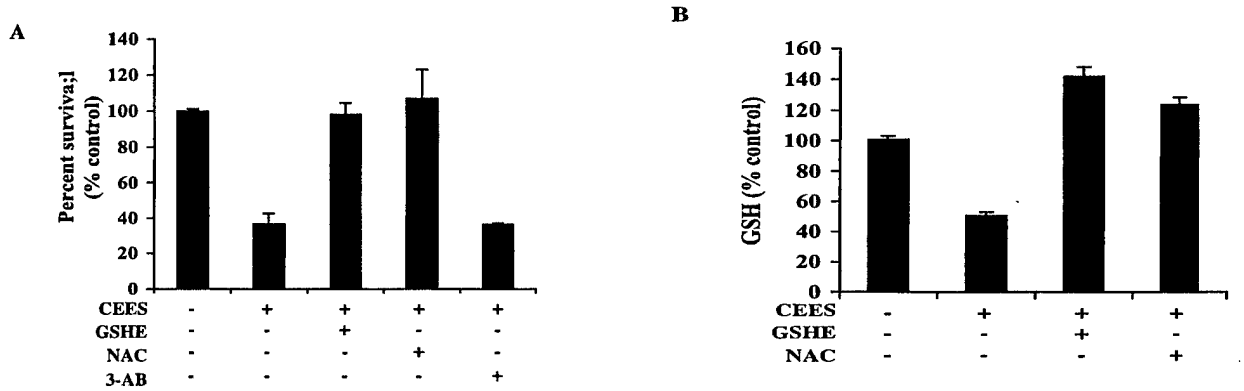


Jurkat cells were first loaded for 1 h with 1 mM GSH-ethyl ester (GSHE) or 5mM NAC and then incubated in the presence or absence of CEES for 5 h, after which cells were incubated with H2DCF probe and then the fluorescence, was measured immediately. In all data above are expressed are means ± S.D. of three independent experiments. *Significant difference in DCF fluorescence was assessed using Student's *t* test ($p < 0.05$).

Protective effect of thiol antioxidants on apoptotic death in Jurkat cells as occasioned by CEES

The data shown above indicated that CEES lowered intracellular GSH levels and that significant increase in ROS levels also resulted by this treatment to Jurkat T cells. Accordingly, it was logical to test whether the co-incubation of selective anti-oxidants would negate the above effects, which were contributing to cell death in Jurkat cells. We thus determined whether thiol anti-oxidants such as GSH and NAC exert a protective effect against cell death induced by CEES.

Figure 3 ***Influence of thiol antioxidants (GSH and NAC) on CEES-induced apoptosis.***



Jurkat T cells were incubated first for 1 h in the presence or absence of 5 mM NAC, 1 mM GSH-ethyl ester or 5 mM 3-AB, and then for 18 h with or without 600 uM CEES in the continued presence or absence of test agent. Cell viability was then assessed by measurement of calcein-AM fluorescence.

Jurkat T cells were initially incubated for 1 h in the presence or absence of 5 mM NAC or 1 mM GSH-ethyl ester and then for 3 h with or without CEES in the continued absence or presence of test agent. Cell extracts were then assayed for GSH. Data are means ± SD of triplicates from a representative experiment. *Significant difference from control on values

In the experiment shown in Figure 3A above, Jurkat cells were incubated in the presence or absence of 5mM NAC for 1 h and subsequently exposed to sulfur mustard half derivative for 18 h (in the continued presence or absence of NAC). It was highly significant that while treatment of Jurkat cells with CEES alone induced >60% cell death, **the presence of NAC totally protected the cells against cell death**. These data as well as similar data using the GSH-ethyl ester rather than NAC indicate that these agents might be useful in the human as a protective and non-toxic measure to overcome potential inflammatory and vesicating activities of sulfur mustard during human exposure. *The use of the Jurkat Cell model, for its experimental convinience will be translated in the second year to kerotinocytes and fibroblasts as well as mouse skin to test these hypotheses. Nevertheless, T-cells have been shown to be affected in individuals who have inhaled large quantities of SM, validating use of Jurkat cells in these preliminary experiments.* The data below will indicate that part of the protective effects of both these anti-oxidants was involved in the protection against the very

early stages of apoptotic activation such as caspase-3 and PARP cleavage. *It also should be noted that caspase-3 activity and PARP cleavage assays have been included in the original SOW in sections 1 and 2 of the amended application as well as in other aspects of the various objectives that were included in the SOW of the original grant.*

Does inhibition of PARP protect cells against sulfur mustard in a similar fashion to anti-oxidants?

Using either mouse fibroblasts, stably transfected with PARP anti-sense cDNA, or fibroblasts derived from PARP knockout mice, we earlier observed that in order to initiate the apoptotic pathway utilizing anti-*Fas* and cycloheximide as inducers, a transient early "burst" of poly ADP-ribosylation was essential (Simbulan-Rosenthal, et al., 1998). Accordingly, we examined the effect of the PARP inhibitor 3-AB on CEES–induced cell death (Figure 3A, 3B shown above). In Jurkat cells exposed to CEES we did not find a major effect of PARP inhibition by 3-AB for protection against CEES toxicity as was noted above due to the thiol anti-oxidants, GSH-ethyl ester and NAC.

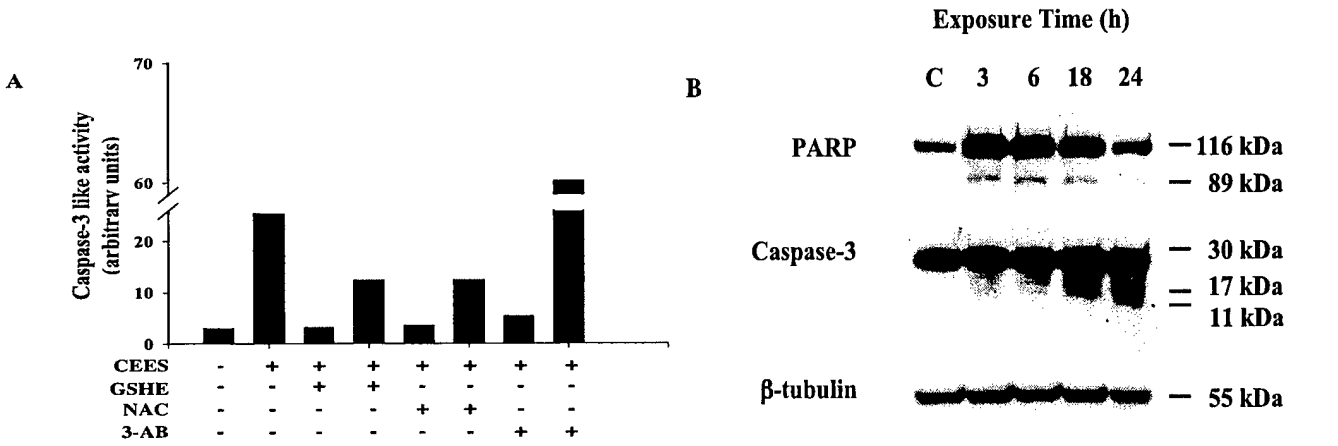
We then wished to determine whether NAC and GSH-mediated protection against CEES cytotoxicity is a result of prevention of a decrease in intracellular GSH levels. Jurkat T cells were therefore incubated in the presence or absence of 5 mM NAC or 1 mM GSH-ethyl ester for 1 h and then exposed to CEES for an additional 6 h (in the continued absence or presence of NAC or GSH-ethyl ester). Treatments with NAC and GSH-ethyl ester completely blocked CEES-induced decrease in GSH levels as shown above (Figure 3B). This data further supports the hypothesis that CEES cytotoxicity is mediated by an increase in ROS and a consequent decrease in intracellular GSH levels.

CEES–induced activation of caspase-3 by both thiol antioxidants and 3-AB.

Caspase-3 is one of the early executioner proteases, which commits cells towards the apoptotic death cascade. Among other proteins, it has been characterized by its ability to cleave PARP at aspartic acid 218, which is located adjacent to the end terminus DNA binding domain of PARP; thus separating PARP into an 89 kDA fragment which contains a modification and activation domain and the 24 kDA DNA binding domain. As noted, PARP is only active catalytically when bound to DNA strand breaks. Since it appeared that CEES

was in part causing cell death due to generation of ROS and general oxidative stress on cells causing DNA strand breaks, we determined whether the protective effect of the thiol antioxidants studies above against CEES toxicity in Jurkat T cells was associated with inhibition of caspase-3 activation from its precursor status. Accordingly, we measured caspase-3–like activity in extracts prepared from cells that had been treated with CEES either alone or in combination with NAC or GSH-ethyl ester. NAC and GSH-ethyl ester each greatly reduced the extent of the increase in caspase-3–like activity induced by CEES as shown below (Figure 4A), a result which is also consistent with the ability of thiol antioxidants to inhibit apoptosis in other cellular systems (Hour *et al.* 1999; Li *et al.* 2000).

Figure 4. Influence of thiol antioxidants on activation of caspase-3 by CEES incubation.



Jurkat T cells were incubated first for 1 h in the presence or absence of 5 mM NAC, 1 mM GSH-ethyl ester, or 5 mM 3-AB, and then for 12 h in the continued presence or absence of test agent. Cell extracts were then prepared and assayed for caspase-3–like activity. Data are expressed in arbitrary units and are from a representative experiment.

Time course study on CEES-induced proteolytic cleavage of PARP and caspase-3 by Western blot analysis. β-tubulin was used as a sample loading control.

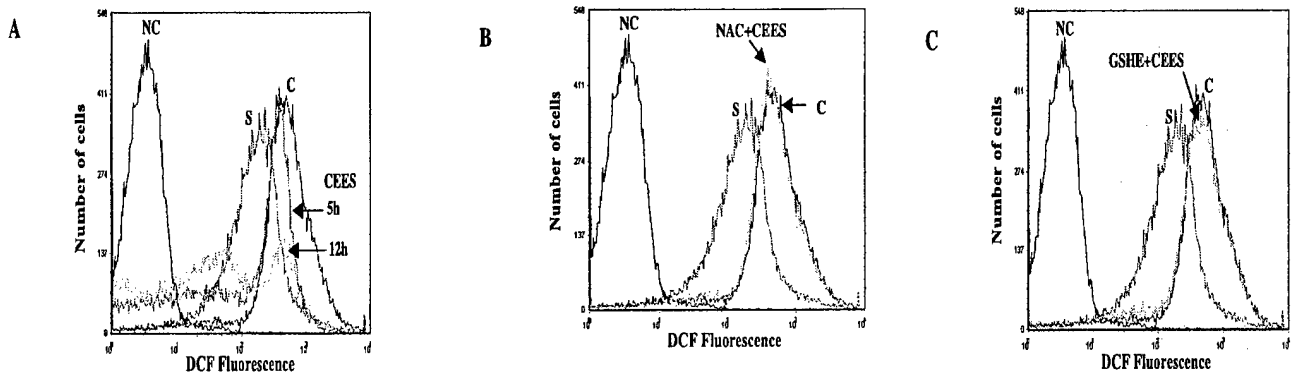
Consistent with the earlier results shown above in Figure 3A, inhibition of PARP activity did not affect the induction of caspase-3 activation.

A direct examination by immunoblotting of CEES (600 uM) effect on activity of caspase-3 is shown by the experiment in Figure 4B above during a 24 h incubation period of Jurkat cells with CEES. As early as 3 hours, some PARP can be detected to be cleaved to the 89 kDa fragment that continued progressively between 17 and 24 h. There was approximately 50% of PARP totally cleaved by caspase-3 as caused by CEES incubation and presumably its ROS generating activities. Additionally, CEES also induces the cleavage of procaspase-3 into its catalytically active p17/p20 form as shown by the Middle Western Blot of figure 4B.

Direct influence of CEES incubation of Jurkat cells on mitochondrial membrane potential ($\Delta\Psi_{mito}$) in vivo

Since the major thrust of part 3 of the aims in the first year's proposal concerning mitochondrial changes these following experiments directly address this aspect of the project. The loss of Ψ_{mito} and hence mitochondrial dysfunction has been observed to accompany apoptotic cell death in various cells (Mancini *et al.*, 1997; Salvioli *et al.*, 1997). The opening of mitochondria permeability transition pores during loss of $\Delta\Psi_{mito}$ has been established to lead to the release of several pro-apoptotic factors, including cytochrome c. This in turn contributes to the activation of caspase-9 (in the proposed experiments described above for number 2 although little progress was made on this topic because of issues already addressed. Caspase-9 was one of the topics to be investigated, albeit in fiberblasts, which we had difficulties in growing during this period with personnel changes) , which then with a number of other factors converts pre-caspase-3 to caspase-3, and the irreversible apoptotic cascade is established (Gottlieb, 2000). Accordingly, we examined whether the sulfur mustard half ester-induced cell death is associated with a loss of $\Delta\Psi_{mito}$. We were also interested in whether the thiol antioxidants, utilized above, prevented this mitochondrial transition. Jurkat T cells were first thus exposed to CEES for 5 h and then $\Delta\Psi_{mito}$ was examined with the specific probe JC-1 by flow cytometry.

Figure 5. Influence of thiol antioxidants on CEES-induced loss of mitochondrial Membrane Potential



Jurkat T cells were incubated for 5 and 12 h in the continued presence or absence of test agent. NC, cells without any treatment and JC-1; S, cells treated with 1 μM of staurosporine; C, control cells. Cells were pre-incubated first for 1 h in the presence or absence of 5 mM NAC.

1 mM GSH-ethyl ester

For 5 h with or without 600uM CEES. Ten minutes before termination of the incubation, the cells were exposed to JC-1after, which $\Delta\Psi_{mito}$ was assessed by flow cytometry.

Figure 5A above shows that exposure of cells to CEES indeed resulted in a slow decrease in $\Delta\Psi_{mito}$. Because the cells exhibited more than one peak at 5 h treatment with CEES, we subsequently increased the exposure time to 12 h, and also for a control used 1 μM of staurosporine, an established inducer of apoptosis, membrane maintenance and lowering of $\Delta\Psi_{mito}$, for 5 h, to exclude that the two peaks were due to a mixture of a possible mixture of viable and some dead cells. At longer exposure with CEES, we observed a marked breakdown of $\Delta\Psi_{mito}$ that is reflected in the increased JC-1 green fluorescence, however the pattern characterized differed from that shown for cells treated with staurosporine. The effects of NAC and GSH-ethyl ester treatment on the loss of $\Delta\Psi_{mito}$ in Jurkat cells was subsequently examined by incubating the cells in the presence or absence of 5 mM NAC or 1 mM GSH-

ethyl ester for 1 h followed by exposure to CEES for 5 h (in the continued presence or absence of NAC or GSH-ethyl ester). The data in Figure 5B demonstrate that **the loss of $\Delta\Psi_{mito}$ by CEES was prevented by the presence of NAC and GSH-ethyl ester (Figure 5C).** *These data support the theory that part of cell toxicity due to sulfur mustard and its derivatives in Jurkat cells is via increased oxidative stress to cells and that the mitochondria per se is a major target of sulfur mustards.*

The data clearly show that the sulfur mustard analog, CEES, has a major effect on $\Delta\Psi_{mito}$, which clearly is associated with the ability of CEES and presumably sulfur mustard *per se* to cause cell death via apoptosis in a number of cells including Jurkat. Thus, we favor the view that the loss of mitochondria membrane potential might be the results in both ROS generation and depletion of intracellular GSH that induce an oxidative modification of the mitochondrial membrane, leading to an osmotic imbalance that can favor apoptosis induction.

Employing a variety of techniques, including a caspase-3 resistant mutant of PARP, our lab previously showed that the proteolytic cleavage of PARP is quite significant for the normal progression of apoptosis in both human osteosarcoma cells and PARP mouse knockout cells (Boulares, et al., 1999, Herceg and Wang, 1999). Blocking this cleavage increases the rate of apoptotic cell death, based upon an excessive depletion of the PARP substrate NAD. It was additionally demonstrated (Boulares, et al., 1999, Herceg and Wang, 1999) that expression of a caspase-3 resistant PARP mutant alters cell death induced by TNFα to both an increase rate of apoptosis as well as necrosis. By reducing excessive depletion of energy reserves such as ATP and NAD and a switch to necrosis, cells exposed to inducers of apoptosis cleave PARP rapidly into inactive peptides and inactivate the enzymatic activity and recombinant depletion of NAD and ATP. In the current experiments however, inhibition of PARP by 3-AB did not protect CEES cells against toxicity nor did it prevent the loss of $\Delta\Psi_{mito}$ induced by this sulfur mustard analog. Nevertheless, our data strongly implicates mitochondrial stress in CEES toxicity, presumably by generation of high levels of ROS, and furthermore demonstrates that CEES-induced loss of $\Delta\Psi_{mito}$ is prevented by treatment of cells with thiol antioxidants. These observations, thus, may provide the development of rational new strategies to protect and potentially prevent the toxic effects of sulfur mustard on both cells and especially tissues such as skin

KEY RESEARCH ACCOMPLISHMENTS

- The biochemical changes characteristic of apoptosis and subsequent mechanism of CEES toxicity in Jurkat T cells has been further investigated in order to associate and identify rational, therapeutic, protective mechanisms against this toxicity.
- A significant reduction in intracellular concentrations of GSH was observed in Jurkat T cells almost immediately after exposure to CEES. This effect was accompanied by increased intracellular ROS generation and by loss of mitochondrial membrane potential that declined slowly at 5 h and more markedly by 12 h. Additionally, L-BSO, an inhibitor of an early step of GSH synthesis, increased CEES induced cell death.
- Additionally, CEES exposure in Jurkat cells induces biochemical markers of apoptotic cell death such as: PARP cleavage, caspase-3 processing and activity.
- The prooxidant effect of CEES was inhibited by either exogenously added thiol-containing antioxidant NAC or GSH-ethyl ester. Accordingly, CEES toxicity appears to be mediated, in part, by generation of ROS and depletion of GSH.
- Since sulfur mustard is still a potentially active biohazard, the protective effects of the various antioxidants against certain types of toxicity of a derivative of this agent in Jurkat cells may be useful to provide approaches to counteract mustard toxicity.

REPORTABLE OUTCOMES

(Research From May 1, 2002- April 30,2003)

CHAPTERS, ABSTRACTS, PRESENTATIONS, AND MANUSCRIPTS

Boulares, A.H., Zoltoski, A.J., Sherif, Z.A., Jolly, P., Massaro, D., **Smulson, M.E.**
Gene knockout or pharmacological inhibition of poly(ADP-ribose) polymerase-1 prevents lung inflammation in a murine model of asthma.
Am J Respir Cell Mol Biol. 2003 Mar;28(3):322-9.

Trofimova, I., Dimtchev, A., Jung, M., Rosenthal, D., Smulson, M., Dritschilo, A., Soldatenkov, V._Gene therapy for prostate cancer by targeting poly(ADP-ribose) polymerase. *Cancer Res.* 2002 Dec 1;62(23):6879-83.

Mandir, A.S., Simbulan-Rosenthal, C.M., Poitras, M.F., Lumpkin, J.R., Dawson, V.L., **Smulson, M.E.,** Dawson, T.M. A novel in vivo post-translational modification of p53 by PARP-1 in MPTP-induced parkinsonism. *J Neurochem.* 2002 Oct;83(1):186-92.

Boulares,A H., Contreras, F., Espinoza, L., and **Smulson, M**. Roles of glutathione and oxidative stress in JP-8 jet fuel-induced apoptosis in rat lung epithelial cells. (2002). T*oxicol. Appl. Pharmacol.* **180:** 92-99.

Rosenthal, D.S., Simbulan-Rosenthal, C.M., Valena, A., Anderson, D., Benton, B., Wang, Z.Q., Smith, B., Ray, R., & **Smulson, M.E**. PARP determines the mode of cell death in skin fibroblasts but not in keratinocytes exposed to sulfur mustard. *J.Invest.Dermatol.* 117(6): 1566-1573 (2001)

Boulares,A.H. ,Zoltoski, A.J.,Zaki, A. S, Yakovlev, A., and **Smulson, M. E.** Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase 1 in sensitization of fibroblasts to tumor necrosis factor-induced apoptosis *Biochem. Biophys. Res. Com.,* 290:796-801(2002)

Boulares, A.H., Zoltoski, A.J., Contreras, F.J., Yakovlev, A.G., Yoshihara, K., **Smulson, M.E**. Regulation of DNAS1L3 Endonuclease Activity by Poly(ADP-ribosyl)ation during Etoposide-induced Apoptosis. *J. Biol. Chem.*, 277(1):372-378 (2002)

Soldatenkov, VA., Chasovskikh, S., Potaman, V.N., Trofimova, I., **Smulson, M.E.**, Dritschilo, A.Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J. Biol. Chem.* ;277(1):665-670. (2002).

CONCLUSIONS

I have made excellent progress on setting up a new cell system (Jurkat cells) and identifying a profitable research direction looking at the role of oxidant related cell injury by mustards. For the second year of the Contract, after a recent site visit with Dr. William Smith, my COR, I plan to extend the research currently being pursued to determine if the protection caused by SM of ROS damage in Jurkat cells will be also seen in 1) a respiratory (rat airway epithelium) model, and 2) a skin (keratinocyte cell line, probably NCO or HaCat) model.

Additionally, during the second year I will be directing my research to points of study that apply to PARP and continue, as in the past, to elucidate if PARP and poly ADP-ribosylation has an active role in the mechanisms of SM pathology, I was lead to appreciate that the USAMRAA had been quite interested in studies which **had a high likelihood of providing direct protection against the pathology to military personal exposed to SM.** At this same time I had begun experiments, which seemed initially quite promising, and indicated that SM was, in part causing a significant increase in cellular Reactive Oxygen species (ROS). Preliminary experiments indicated that natural antioxigents seemed to protect cells against SM –induced death. After discussions with my COR, we began to direct most of our SOW to this topic for year one of the contract. It should be noted that several of the original objectives of the amended Contract were, in fact, directly related to ATP/NAD levels in cells, and stresses placed on mitochondria membrane potentials; nicely complements this new work initiated in year one.

Since at that time, we also began to experience difficulties with the growth of our various skin cells, which had been used successfully in the past.

SM induced ROS, protection by Antioxidants

Accordingly, we began a detailed study on Protective roles of ant-oxidants in Jurkat T-cells on sulfur half mustard (CEES). A manuscript on the first year's work on this topic was recently submitted to *The British Journal of Pharmacology*, and the data from this paper constitute the bulk of the Report for this first year. Essentially, in this work, we investigated the biochemical changes characteristic of apoptosis and subsequent mechanism of CEES toxicity in Jurkat T cells in order to associate and identify rational, therapeutic, protective mechanisms against this toxicity. A

significant reduction in intracellular concentrations of GSH was observed in Jurkat T cells almost immediately after exposure to CEES. This effect was accompanied by increased intracellular ROS generation and by loss of mitochondrial membrane potential that declined slowly at 5 h and more markedly by 12 h. Also, L-BSO, an inhibitor of an early step of GSH synthesis, increased CEES induced cell death.

Additionally, CEES exposure in Jurkat cells induced biochemical markers of apoptotic cell death such as: PARP cleavage, caspase-3 processing and activity. The prooxidant effect of CEES was inhibited by either exogenously added thiol-containing antioxidant NAC or GSH-ethyl ester. Accordingly, CEES toxicity appears to be mediated, in part, by generation of ROS and depletion of GSH. Since sulfur mustard is still a potentially active biohazard, the protective effects of the various antioxidants against certain types of toxicity of a derivative of this agent in Jurkat cells may be useful to provide approaches to counteract mustard toxicity.

"SO WHAT?"

We believe this work is of importance since the perturbation of mitochondrial function by a SM derivative during earlier apoptosis increased generation of reactive oxygen species (ROS) which results in an oxidative environment that contributes to the apoptotic death process, in part, by depleting cellular antioxidants such as reduced glutathione (GSH) and by inducing direct damage to DNA, proteins, and lipids. A rationale supporting this biological mechanism is based on past studies with other toxic agents, the results would indicate that thiol anti-oxidants are highly effective in rescuing cells from toxic induced cell death. Given that the toxicity of SM and concomitant generation of ROS has been proposed to result from electrophilic or oxidative stress with depletion of cellular detoxifying thiol levels including glutathione, we have now examined whether CEES induces toxicity as well as apoptosis in Jurkat T cells by inducing the generation of free radicals and, hence, affecting cellular antioxidant systems The effects of oxidative stress on CEES toxicity in Jurkat T cells as well as the possible protective properties of GSH and NAC against CEES were additionally investigated with respect to cell death.

As noted earlier for the second year of the Contract, after a recent site visit with Dr.. William Smith (my COR), I plan to extend the research currently being pursued to determine if the protection caused by SM of ROS damage in Jurkat cells will be also seen in 1) a respiratory (rat airway epithelium) model, and 2) a skin (keratinocyte cell line, probably NCO or HaCat) model.

Additionally, during the second year I will be directing my research to points of study that apply to PARP and continue, as in the past, to elucidate if PARP and poly ADP-ribosylation has an active role in the mechanisms of SM pathology,

Key Words: 2-chloroethylethyl sulfide, sulfur mustard, apoptosis, NAC, ROS, mitochondrial membrane potential, poly (ADP-ribose) polymerase

APPENDIX

Annual Report, May 30,2003

Molecular Biology Basis for the Response of Poly(ADP-rib) polymerase and NAD
Metabolism to DNA Damage Caused by Mustard Alkylating Agents.
DAMD17-02-C-0085

Principle Investigator: Mark E. Smulson, Ph.D.
Professor of Biochemistry and Molecular Biology
Georgetown University School of Medicine

Contents:

1. **Experimental Methods**
2. **References**
3. **Curriculum Vitae**

APPENDIX

1) METHODS USED FOR EXPERIMENTS DESCRIBED IN ANNUAL REPORT

Materials

Fetal bovine serum was obtained from Quality Biological, INC. (Gaithersburg, MD, U.S.A.). RPMI 1640 was obtained from Invitrogen (Carlsbad, CA, U.S.A.). GSH-ethyl ester, NAC, L-BSO, hydrogen peroxide and anti-β-tubulin were purchased from Sigma Chemical Company (St, Louis, MO, U.S.A.). CEES was obtained from Aldrich (Milwaukee, WI, U.S.A.). Calcein-AM, JC-1 and H2DCF were obtained from Molecular probes (Eugene, OR, U.S.A.) and anti-PARP from BD Transduction Laboratories (San Diego, CA, U.S.A.). Anti-mouse IgG peroxidase-conjugated secondary antibody was purchased from Amersham (Piscataway, NJ, U.S.A.) and anti-caspase-3 from Santa Cruz (Santa Cruz, CA, U.S.A.). Ac-DEVD-AMC was obtained from Biomol (Plymouth Meeting, PA, U.S.A.).

Cell culture and treatment

The Jurkat T cell line was maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated serum (v/v), penicillin (100 U/ml) and streptomycin (100 mg/ml). A 14% (v/v) CEES solution was prepared in absolute ethanol and was further diluted 1:2000 with complete growth medium. A 0.05% solution of ethanol in culture medium (vehicle) had no significant effect on cell viability (data not shown). The density of CEES is 1.07 g/ml; a 1/14,000 dilution therefore corresponds to a CEES concentration of 600 μM.

Cell viability assay

For determination of cell viability, cells were seeded in 24-well plates. After treatment with 600 μM CEES, cells were exposed to calcein-AM (Molecular Probes, Eugene, OR) to a final concentration of 2.5 μM and then

the plate was incubated for 30 min at 37°C. Fluorescence resulting from the deesterification of calcein-AM was monitored with a CytoFluor 4000 fluorometer (PerSeptive Biosystems, Framingham, MA) at excitation and emission settings of 488 and 520 nm, respectively.

Assay for intracellular GSH

Cells were incubated with CEES at previously indicated concentrations and immediately washed twice with PBS. The cell pellets were then lysed in 10 mM HCl by freezing and thawing three times followed of deproteinization with 10% 5-sulfosalicylic acid and centrifuged at 1000 x g for 5 min. The supernatant solution was assayed for nonprotein sulfhydryls by quantifying, using a spectrophotometer, the reduction of DNTB (5,5'-dithio-bis-[2-nitrobenzoic acid]) through its conversion to 5-thio-2-nitrobenzoic acid at 412 nm. Standard curves were run in all experiments with known amounts of GSH.

Caspase-3 activity measurement

Caspase-3 activity was assessed using z-Asp-Glu-Val-Asp (DEVD) as a substrate in the established fluorescent assay. Cell extracts (30 μg of protein) were incubated with 40 mM DEVD-AMC peptide substrate in a total volume of 200 μl. The free aminomethylcoumarin (AMC) fluorescence, which is produced by the cleavage of the aspartate–AMC bond, was measured continuously utilizing a CytoFluor 4000 fluorometer at excitation and emission wavelengths of 360 and 460 nm, respectively for 30 min. The fluorescent emission from each well was plotted versus time, and linear regression analysis of the initial velocity for each curve yielded the activity.

Measurement of ROS levels

The assay for ROS measure was monitored using the cell-permeant probe H2DCF. Cells growing on 96-well culture plates were pretreated with CEES for 5 h. Then cells were loaded with H2DCF by addition of the diacetate form of this compound (Molecular Probes) to the medium at a final concentration of 10 μM in the dark for 15 min. In the presence of ROS, the nonfluorescent dichlorodi-hydrofluorescein is oxidized to the

highly fluorescent 2,7-dichlorofluorescein. Fluorimetric analysis was measured with an excitation of 485 nm and emission of 530 nm.

Mitochondrial Membrane Potential Measurement ($\Delta\Psi_{mito}$)

After treatment with CEES, cells were assayed for mitochondrial membrane potential with 5,5',6,6',-tetrachloro-1,1'3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes), a cell-permeable dye that becomes concentrated in the mitochondria and generates red fluorescence in mitochondria with a high $\Delta\Psi_{mito}$. After CEES exposure for 5 h, cells were incubated for 10 min at 37°C with 15 μg/ml of JC-1, and then analyzed for loss of JC-1 fluoresce as $\Delta\Psi_{mito}$ using a Becton Dickinson FACS flow cytometer (Boulares *et al.*, 2001).

Immunoblot Analysis

Cells were harvested, washed with ice–cold phosphate-buffered saline. Cells were lysed in ice-cold lysis buffer containing (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenylmethylsulphonylfluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 20 μg/ml leupeptin. Thirty micrograms of protein per lane was subjected to SDS-PAGE on 4-20% gradient gels, followed by transfer to nitrocellulose membrane and incubation with the first antibodies overnight. Proteins were visualized with horseradish peroxidase-conjugated anti-mouse followed by use of ECL chemilomuniscence kit (Pierce, Rockgold, IL).

Statistical analysis

All statistical analyses to determine the significances of differences between experimental conditions were performed using the Student's *t* test. A difference of $P < 0.05$ was

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3 CV

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. DO NOT EXCEED FOUR PAGES.

NAME	POSITION TITLE
Mark E. Smulson	Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUD
Washington & Lee University, Lexington, VA	AB	1958	Chemistry
Cornell University, Ithaca, NY	MS	1961	Nutrition
Cornell University, NY	PhD	1964	Biochemistry
Einstein Medical Center, Philadelphia, PA	Post-doc	1965-65	Nucleoside Analogs
NIH, Bethesda, MD	Post-doc	1966-67	Tumor Molecular Bi

A. POSITIONS AND HONORS

Positions

1967-1972 Assistant Professor, Biochemistry, Georgetown University Medical School

1972-1978 Associate Professor of Biochemistry, Georgetown University Medical School

1978 - present Professor of Biochemistry and Molecular Biology, Georgetown University
1989 - present Lombardi Cancer Center, Co-Director, Program of Radiation Biology

Honors

- Awarded First Annual Dean's Prize for Biomedical Research, 1988
- Meeting Organizer International Meeting on Poly(ADP-Rib), 6/73 at NIH.
  - Organizer Princess Takamatsu Cancer Meeting, Tokyo, Japan (9/82).

B. REPRESENTATIVE PUBLICATIONS (SELECTED 1995-2002) [37 OUT OF 128]

Cynthia M. Simbulan-Rosenthal*, Dean S. Rosenthal, RuiBai Luo, Raed Samara, Luis A. Espinoza, Paul O. Hassa†, Michael O. Hottiger†, and **Mark E. Smulson** PARP-1 binds E2F-1 independently of its DNA binding and catalytic domains, and acts as a novel coactivator of E2F-1-mediated transcription during reentry of quiescent cells into S-phase, *Oncogene* (in Press). 2003

Espinoza, L.A., **Smulson, M.E.** Macroarray analysis of the effects of JP-8 jet fuel on gene expression in Jurkat cells. *Toxicology* (In press) 2003

Han, S., Espinoza, L.A., Boulares, A.H. and Smulson, M.E. Protection by antioxidants against toxicity and apoptosis induced by the sulfur mustard analog CEES (2-chloroethylethyl sulfide) in Jurkat T cells (submitted) 2003

Trofimova, I., Dimtchez, A., Jing, M., Rosenthal, D.S., **Smulson, M.E.**, Dritschilo, A., and Soldatenkov, V., Gene Therapy for Prostate Cancer by Targeting Poly(ADP-Ribose) Polymerase. *Cancer Res.* (In Press) (Dec 1 2002)